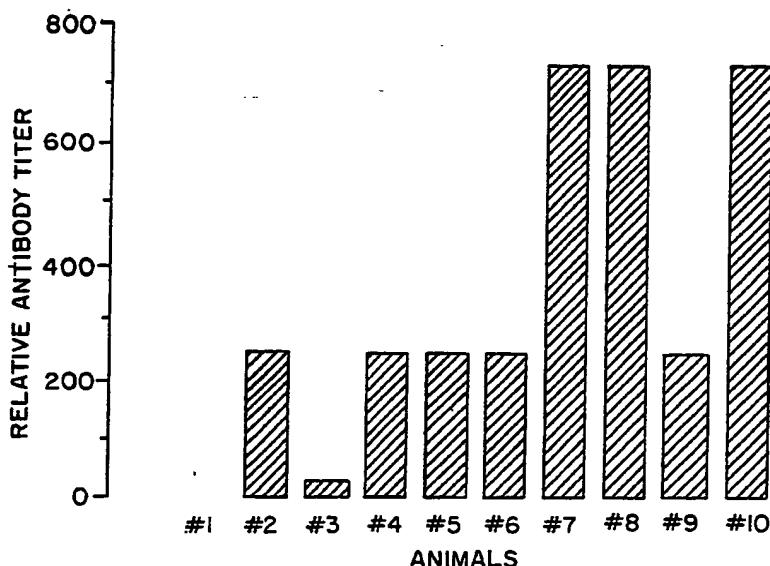




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :  A61K		(11) International Publication Number: <b>WO 92/20316</b>
	A2	(43) International Publication Date: 26 November 1992 (26.11.92)
(21) International Application Number: PCT/US92/03875		(74) Agents: DECONTI, Giulio, A., Jr. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).
(22) International Filing Date: 14 May 1992 (14.05.92)		
(30) Priority data: 699,891 14 May 1991 (14.05.91) US		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).
(71) Applicants: UNIVERSITY OF CONNECTICUT [US/US]; Storrs, CT 06269 (US). TARGETECH, INC. [US/US]; 290 Pratt Street, Meriden, CT 06450 (US).		
(72) Inventors: WU, George, Y.; 15 Rundelane, Bloomfield, CT 06002 (US). SPITALNY, George, L.; 6 Brookfield Court, Cheshire, CT 06410 (US). FINDEIS, Mark, A.; 234 Sherman Avenue, Apt. #29, Meriden, CT 06450 (US).		Published <i>Without international search report and to be republished upon receipt of that report.</i>

## (54) Title: TARGETED DELIVERY OF GENES ENCODING IMMUNOGENIC PROTEINS



## (57) Abstract

Molecular complexes can be used to target a gene encoding an immunogenic protein or polypeptide to a specific cell *in vivo*. The gene is incorporated into the target cell, expressed and the gene-encoded product is secreted and an immune response against the immunogenic protein or polypeptide is elicited. The molecular complex comprises an expressible gene encoding a desired immunogenic protein or polypeptide complexed with a carrier of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent is specific for a cellular surface structure which mediates internalization of ligands by endocytosis. An example is the asialoglycoprotein receptor of hepatocytes. The gene-binding agent is a compound such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can function within a cell. The molecular complex is stable and soluble in physiological fluids and can be used to elicit an immune response against a variety of immunogens in an organism for the purpose of vaccination or for the production of antibodies.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LJ	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		

-1-

TARGETED DELIVERY OF GENES  
ENCODING IMMUNOGENIC PROTEINS

Background of the Invention

Immunization of animals for the purpose of  
05 vaccination or production of antibodies (used for  
passive immunization, diagnostic or scientific  
reagents) typically involves injection of a natural  
or recombinant protein which has been partially or  
completely purified to homogeneity. Purification of  
10 the protein to homogeneity usually requires several  
steps involving anionic, cationic and molecular sieve  
chromatography. These procedures are time consuming  
and the homogeneity of the final product must be  
verified. Alternatively, a differentiating assay  
15 which can distinguish the protein of choice from all  
other proteins can be used to develop a monoclonal  
antibody.

Summary of the Invention

This invention pertains to a soluble molecular  
20 complex for targeting, a gene (or genes) encoding an  
immunogenic protein or polypeptide for which an  
immune response is desired, to a specific cell in a  
host organism in vivo and obtaining expression of the  
gene, production of the gene-encoded protein or  
25 polypeptide and development of an immune response  
against the immunogenic protein or polypeptide in the  
host organism. The molecular complex comprises a

nucleic acid molecule containing an expressible gene encoding a desired immunogenic protein or polypeptide complexed with a carrier which is a conjugate of a cell-specific binding agent and a gene-binding 05 agent. The cell-specific binding agent is specific for a cellular surface structure, typically a receptor, which mediates internalization of bound ligands by endocytosis, such as the asialoglycoprotein receptor of hepatocytes. The 10 cell-specific binding agent can be a natural or synthetic ligand (for example, a protein, polypeptide, glycoprotein, etc.) or it can be an antibody, or an analogue thereof, which specifically binds a cellular surface structure which then 15 mediates internalization of the bound complex. The gene-binding component of the conjugate is a compound such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can 20 function within the cell.

The complex of the gene and the carrier is stable and soluble in physiological fluids. It can be administered in vivo where it is selectively taken up by the target cell via the surface-structure-mediated endocytotic pathway. The incorporated gene is expressed, the gene-encoded protein or polypeptide is processed and secreted as a soluble or a cell surface protein or polypeptide by the transfected cell and an immune response is evoked against the 25 30 protein or polypeptide in the host organism.

The soluble molecular complex of this invention can be used to elicit an immune response in an organism to a desired immunogenic protein or

polypeptide. It can be used for immunization of organisms for the purpose of vaccination or for the production of antibodies for experimental (e.g., research reagent), diagnostic or therapeutic use.

05 Brief Description of the Figure

Figure 1 is a bar graph representing the relative titer of anti-HBsAg antibody in animals receiving the molecular complex of the invention.

Detailed Description of the Invention

10 A soluble, targetable molecular complex is used to deliver a gene encoding an immunogenic protein or polypeptide to a target cell or tissue in vivo and obtain expression of the gene, production of the gene-encoded protein or polypeptide and development  
15 of an immune response against the immunogenic protein or polypeptide in a host organism. The molecular complex comprises the gene encoding a desired immunogenic protein or polypeptide to be delivered complexed with a carrier made up of a binding agent  
20 specific for the target cell and a gene-binding agent.

The gene, generally in the form of DNA, encodes the desired immunogenic protein or polypeptide. Typically, the gene comprises a structural gene encoding the immunogenic protein or polypeptide in a  
25 form suitable for processing and secretion as a soluble or cell surface protein or polypeptide by the target cell. For example, the gene encodes appropriate signal sequences which direct processing and secretion of the protein or polypeptide. The  
30 signal sequence may be the natural sequence of the protein or exogenous sequences. The structural gene

-4-

is linked to appropriate genetic regulatory elements required for expression of the gene-encoded protein or polypeptide by the target cell. These include a promoter and optionally an enhancer element operable 05 in the target cell. The gene can be contained in an expression vector such as a plasmid or a transposable genetic element along with the genetic regulatory elements necessary for expression of the gene and secretion of the gene-encoded product.

10 The carrier component of the complex is a conjugate of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent specifically binds a cellular surface structure which mediates internalization by, for example, the process 15 of endocytosis. The surface structure can be a protein, polypeptide, carbohydrate, lipid or combination thereof. It is typically a surface receptor which mediates endocytosis of a ligand. Thus, the binding agent can be a natural or synthetic 20 ligand which binds the receptor. The ligand can be a protein, polypeptide, glycoprotein or glycopeptide which has functional groups that are exposed sufficiently to be recognized by the cell surface structure. It can also be a component of a 25 biological organism such as a virus, cells (e.g., mammalian, bacterial, protozoan) or artificial carriers such as liposomes.

The binding agent can also be an antibody, or an analogue of an antibody such as a single chain 30 antibody which binds the cell surface structure.

Ligands useful in forming the carrier will vary according to the particular cell to be targeted. For targeting hepatocytes, glycoproteins having exposed

terminal carbohydrate groups such as asialoglycoprotein (galactose-terminal) can be used, although other ligands such as polypeptide hormones may also be employed. Examples of asialoglycoproteins include

05 asialoorosomucoid, asialofetuin and desialylated vesicular stomatitis virus. Such ligands can be formed by chemical or enzymatic desialylation of glycoproteins that possess terminal sialic acid and penultimate galactose residues. Alternatively,

10 asialoglycoprotein ligands can be formed by coupling galactose terminal carbohydrates such as lactose or arabinogalactan to non-galactose bearing proteins by reductive lactosamination.

For targeting the molecular complex to other

15 cell surface receptors, other types of ligands can be used, such as mannose for macrophages, mannose-6-phosphate glycoproteins for fibroblasts, intrinsic factor-vitamin B12 for enterocytes and insulin for fat cells. Alternatively, the cell-specific binding

20 agent can be a receptor or receptor-like molecule, such as an antibody which binds a ligand (*e.g.*, internalizing antigen) on the cell surface. Such antibodies can be produced by standard procedures.

The gene-binding agent complexes the gene to be

25 delivered. Complexation with the gene must be sufficiently stable *in vivo* to prevent significant uncoupling of the gene extracellularly prior to internalization by the target cell. However, the complex is cleavable under appropriate conditions

30 within the cell so that the gene is released in functional form. For example, the complex can be labile in the acidic and enzyme rich environment of lysosomes. A noncovalent bond based on electrostatic

attraction between the gene-binding agent and the gene provides extracellular stability and is releasable under intracellular conditions.

Preferred gene-binding agents are polycations 05 that bind negatively charged polynucleotides. These positively charged materials can bind noncovalently with the gene to form a soluble, targetable molecular complex which is stable extracellularly but releasable intracellularly. Suitable polycations are 10 polylysine, polyarginine, polyornithine, basic proteins such as histones, avidin, protamines and the like. A preferred polycation is polylysine. Other noncovalent bonds that can be used to releasably link the expressible gene include hydrogen bonding, 15 hydrophobic bonding, electrostatic bonding alone or in combination such as, anti-polynucleotide antibodies bound to polynucleotide, and strepavidin or avidin binding to polynucleotide containing biotinylated nucleotides.

20 The carrier can be formed by chemically linking the cell-specific binding agent and the gene-binding agent. The linkage is typically covalent. A preferred linkage is a peptide bond. This can be formed with a water soluble carbodiimide as described 25 by Jung, G. *et al.* (1981) Biochem. Biophys. Res. Commun. 101:599-606. An alternative linkage is a disulfide bond.

The linkage reaction can be optimized for the particular cell-specific binding agent and 30 gene-binding agent used to form the carrier. Reaction conditions can be designed to maximize linkage formation but to minimize the formation of aggregates of the carrier components. The optimal

ratio of cell-specific binding agent to gene-binding agent can be determined empirically. When polycations are used, the molar ratio of the components will vary with the size of the polycation and the 05 size of the cell-specific binding agent. When a protein such as asialoorosomucoid and a polycation such as polylysine are used, the mass ratio of protein to polycation will typically be in the range of 5:1 to 1:5, preferably around 1:1. The molar 10 ratios in these mixtures will vary widely with the molecular weights of the components. For the same protein, a greater number of molar equivalents of a lower molecular weight polycation will be required to prepare a conjugate with the same cation content as a 15 conjugate prepared with a higher molecular weight polycation. Uncoupled components and aggregates can be separated from the carrier by molecular sieve chromatography or ion-exchange chromatography or a combination of the two techniques.

20 The gene encoding the desired immunogenic protein or polypeptide can be complexed to the carrier by combining these two components in solution containing NaCl at a concentration of 0.15 to 2 M. The carrier is added to the DNA and if a higher NaCl 25 concentration is used, the solution of complex is dialyzed to reduce the salt concentration. In a preferred method, the carrier and DNA are both in 0.15 M NaCl and the carrier is added to the DNA to form a complex directly.

-8-

The gene encoding the desired immunogenic protein or polypeptide can be complexed to the carrier by a stepwise dialysis procedure. In a preferred method, for use with carriers made of 05 polycations such as polylysine, the dialysis procedure begins with a 2 M NaCl dialyzate and ends with a 0.15 M NaCl solution. See e.g., Wu, G.Y. and Wu, C.H. J. Biol. Chem. (1987) 262:4429-4432. The gradually decreasing NaCl concentration results in 10 binding of the DNA to the carrier.

The molecular complex can contain more than one copy of the same gene or one or more different genes. Preferably, the mass ratio of carrier to polynucleotide is about 1:5 to 5:1, preferably about 15 2:1. This ratio will vary considerably depending upon the components of the complex. For example, a conjugate with a lower proportion of polycation will typically be used at a higher ratio to the DNA.

The molecular complex of this invention can be 20 administered parenterally. Preferably, it is injected intravenously. The complex is administered in solution in a physiologically acceptable vehicle.

Cells can be transfected in vivo for transient expression and secretion of the gene-encoded 25 product. For prolonged expression and secretion, the gene can be administered repeatedly. Alternatively, the transfected target cell can be stimulated to replicate by surgical or pharmacological means to prolong expression of the incorporated gene. See, 30 for example, U.S. Patent Application Serial No. 588,013, filed September 25, 1990, the teachings of which are incorporated by reference herein.

The molecular complex of this invention is adaptable for delivery of a wide range of genes to a specific cell or tissue. Preferably, the complex is targeted to the liver by exploiting the hepatic 05 asialoglycoprotein receptor system which allows for in vivo transfection of hepatocytes by the process of receptor-mediated endocytosis. The liver has the highest rate of protein synthesis per gram of tissue. Thus, the molecular complex of this 10 invention can be used to specifically target the liver as a site for high efficiency production of an immunogenic protein or polypeptide to thereby elicit an immune response to the protein or polypeptide.

The immunogen is typically, but not necessarily, 15 a protein or polypeptide foreign to the host. It can be any protein or polypeptide which contains an epitope or epitopes (B or T cell), which are immunogenic in a host organism. For vaccination, the immunogen can be an immunogenic component or 20 components of a pathogen, such as a virus, bacterium, or parasite, which can elicit a protective immune response against the pathogen. For example, the immunogen can be an envelope protein of a virus (e.g., HIV glycoprotein, HBV surface antigen) or a 25 cell wall constituent of a bacterium.

In some cases, the protein or polypeptide against which the immune response is desired may be nonimmunogenic or poorly immunogenic. Such proteins or polypeptides can be coupled to an immunogenic 30 carrier protein. This can be accomplished genetically by preparing a chimeric gene that encodes a fusion of the protein or polypeptide and the carrier.

-10-

The immunogen can be any agent against which antibodies are desired for diagnostic or therapeutic purposes. For example, the immunogen can be a component of a pathogen which elicits antibodies 05 and/or cell useful for passive immunization against the pathogen. The immunogen can be a cell surface structure associated with a diseased cell such as tumor-associated antigen which elicits anti-tumor antibodies for diagnosis or therapy of tumor. The 10 immunogen can also be any agent against which antibodies are needed for diagnostic assays. Examples include pathogens, hormones, cytokines, metabolites or drugs.

The method of this invention can be used to 15 vaccinate an organism (human or other animal) to provide protection against infection. In one embodiment, a gene encoding an immunogenic protein or polypeptide which is a component of a pathogen is complexed to a conjugate of an asialoglycoprotein and 20 a polycation. The resulting soluble complex is administered to a host organism to target liver cells in amounts sufficient to selectively transfet the cells and to provide sufficient production and secretion of the immunogen to elicit a protective 25 immune response against the immunogen in the organism.

The method of this invention can also be used to produce polyclonal or monoclonal antibodies. For production of a polyclonal antibody, a gene encoding the immunogenic protein or polypeptide complexed with 30 a carrier is administered to an organism (e.g., mouse, rabbit or goat) to elicit an immune response (antibody and/or cellular) against the immunogenic

protein or polypeptide. Antiserum which is specific for the immunogen is obtained from the immunized organism by known techniques.

For production of monoclonal antibodies, spleen 05 cells or other antibody-producing cells are obtained from an organism immunized with the molecular complex. These cells are fused with appropriate immortalizing cells, such as myelomas, to produce hybridomas. The hybridomas are screened and those 10 which produce antibodies specific for the immunogenic protein or polypeptide are selected.

In vivo gene transfer of an immunogenic protein or polypeptide to immunize an organism has several advantages over injection of a natural or recombinant 15 protein which has been partially or completely purified to homogeneity. Expression and secretion of an immunogenic protein or polypeptide in vivo eliminates the necessity of purifying an immunogen and can invoke a polyclonal antibody response that 20 will be monospecific for the immunogenic protein or polypeptide. In addition, continuous expression and secretion of the immunogenic protein or polypeptide can boost the initial immune response in an organism.

This invention is illustrated by the following 25 exemplification.

#### Exemplification

##### General

Polylysines (PL), obtained as the hydrobromide salts, and 3'dimethylaminopropyl ethyl carbodiimide 30 (EDC) were from Sigma Chemical Co. Acrodisc syringe filters were obtained from Gelman Sciences.

-12-

Orosomucoid was isolated from pooled human plasma as described by Whitehead and Sammons (Biochim. Biophys. Acta (1966) 124:209-211) and desialylated to form asialoorosomucoid (ASOR) by treatment with 0.1 N  $H_2SO_4$  at 80°C for 1 hour (Schmid *et al.* (1967) Biochem. J. 104:361-368). ASOR concentrations were determined using an  $OD_{280}$  of 0.92 for a 1.0 mg/mL solution in water. DNA concentrations were determined using an  $OD_{260}$  of 1.0 for a 50  $\mu$ g/mL solution in water. HPLC purification of ASOR-PL conjugates was performed using a Brownlee Aquapore CX-300 cation exchange column (10 mm x 25 cm) obtained from Rainin Instrument Co. Dialysis tubing was obtained from Spectrum Medical Industries.

15 Synthesis of asialoorosomucoid-polylysine (ASOR-PL) conjugate  
ASOR (60 mg/8 mL), PolyLysine HBr (5200 MW, 92 mg/3 mL) and EDC (42 mg/2 mL) were each dissolved in water. Each solution was filtered (0.45  $\mu$ m) and 20 the filter was washed with 2, 1 and 1 mL of water, respectively, which was combined with the corresponding solution. The pH's of the ASOR and the Polylysine solutions were adjusted to 7.4 with 132  $\mu$ L and 420  $\mu$ L of 0.1 N NaOH respectively. The EDC 25 solution plus a 1 mL rinse was added to the ASOR solution with stirring and the pH of the mixture adjusted to 7.4 with 4  $\mu$ L of 0.1 N NaOH. The polylysine solution plus a 1 mL rinse with water was added with stirring to the ASOR-EDC solution and the 30 pH adjusted to 7.4 with 92  $\mu$ L of 0.1 N NaOH. The reaction mixture was incubated for 24 hours at 37°C. The reaction mixture was then placed in 12,000-14,000

MW cut-off dialysis tubing and dialyzed against 20L of water for 24 hours and then twice against 8L of water for 3 hours. The resulting solution was lyophilized to yield 58 mg of ASOR-PL conjugate

05 (38.0% based on ASOR and polylysine combined masses). Based on the OD<sub>280</sub> of a 1.0 mg/mL solution in water (0.68) this conjugate was determined to be 74% ASOR by weight.

Alternative procedures

10 ASOR-PL conjugates prepared with polylysines of different molecular weights (Mr = 21,000 and 69,000) have also produced DNA complexes that produce similar positive results in animal experiments. These conjugates are synthesized in essentially the same

15 manner as the conjugates made with 5.2Kd polylysine, with the additional step after dialysis of purification by HPLC followed by dialysis and lyophilization. The HPLC purification is performed on a Brownlee Aquapore cation exchange column (10 mm

20 x 25 cm) with a flow rate of 4.5 mL/min. The column was eluted with buffers containing 0.1 M sodium acetate adjusted to acidic pH with HCl. A gradient from pH 5.0 to 2.0 was used to elute unconjugated ASOR, conjugate and polylysine. Conjugates typically

25 elute at pH 2.5 to 2.0. In some runs, conjugate and excess polylysine overlap. In those instances, the front portion of the main peak that is rich in ASOR, as determined by the higher ratio of the absorbances at 280 nm and 230 nm, is collected and used for DNA

30 complex formation as described above.

Gel retardation assays

A plasmid containing a head-to-tail dimer of the complete hepatitis B viral genome citation was made up to 100  $\mu$ g/mL in 0.3 M NaCl and filtered (0.2  $\mu$ m).

05 An aliquot of the DNA solution (5  $\mu$ L, 500 ng DNA) was placed in each of sixteen 1.5 mL polypropylene microtubes. A 1 mg/mL stock of ASOR-PL (5.2Kd polylysine) conjugate in water was prepared and filtered (0.2  $\mu$ m). Solutions of the conjugate 10 diluted with water were added to the DNA samples to create conjugate to DNA ratios in the range from 0.2:1 to 3.0:1 (w/w). Water was added to each sample to bring the final DNA concentration to 50  $\mu$ g/mL and the NaCl concentration to 0.15 M. The samples were 15 vortexed briefly, centrifuged for 30 seconds at 14,000 rpm and incubated at room temperature for 1 hour. Loading buffer (3.3  $\mu$ L of 40% sucrose, 0.25% bromophenol blue) was added to each sample which was then loaded on to a 1% agarose gel. The gel was 20 prepared by dissolving 0.4 g of agarose in 40 mL of TPE buffer (90 mM TRIS-phosphate, 2 mM EDTA, pH 8.0), adding 0.5  $\mu$ L of a 10 mg/mL solution of ethidium bromide. The gel was 8 x 6.5 x 0.7 cm, the running buffer used was TPE buffer and a constant 50 V was 25 applied for 1.5 hours. Extent of retardation of DNA was then observed under UV light. Full retardation was taken as that ratio of conjugate to DNA that retained all of the DNA in the sample well of the gel.

DNA-Conjugate complex formation

To plasmid (870  $\mu$ L of a 0.2  $\mu$ m-filtered solution at 915  $\mu$ g/mL) was added water (523  $\mu$ L) and 4.0 M NaCl to give a final DNA concentration of 500  $\mu$ g/mL and a 05 final NaCl concentration of 0.15-0.5 M. This sample was placed in a 5 mL vial and stirred. To ASOR-PL (5.2Kd polylysine) conjugate (597  $\mu$ L of a 0.2  $\mu$ m filtered solution at 2.0 mg/mL) was added water (796  $\mu$ L) and 4.0 M NaCl (199  $\mu$ L) to give a final conjugate 10 concentration of 750  $\mu$ g/mL and a final NaCl concentration of 0.15-0.5 M. The conjugate was added to the DNA solution at 6.7  $\mu$ L/min (0.4 mL/h) via a peristaltic pump. After complete addition the plasmid DNA/ASOR-PL complex was filtered (0.2  $\mu$ m) and 15 determined by UV to contain 250  $\mu$ g/mL of DNA. Complexation was verified by running an agarose gel of plasmid DNA (500 ng) and the complex (500 ng of DNA).

In vivo transfection of animals and detection of HBsAg

20 BALB/c mice 8-16 weeks of age, purchased from Harlan Sprague Dawley, Inc. (Madison, WI), were injected intravenously with 34  $\mu$ g to 125  $\mu$ g of HBV DNA contained in a DNA/ASOR-PL complex. Fifteen - 30 minutes after injection, mice were anesthetized with 25 ether and a 2/3 partial hepatectomy performed to induce a more prolonged state of gene expression. For this procedure a midline incision was made from just below the sternum extending midway down the abdomen. From the opening in the peritoneal cavity 30 the liver was exposed and surgical silk was wrapped around the major bottom lobe of the liver. The silk was tied off just below the point where the hepatic

-16-

veins join the inferior vena cava. The ligated region of the liver was excised and the incision closed with wound clips.

Serum samples were collected from mice at 05 varying intervals after injection of the DNA/ASOR-PL complex and initially tested for the presence of hepatitis B surface antigen in the circulation using an ELISA kit from Abbott Laboratories (Abbott Park, IL). This test can detect as little as 5 pico grams 10 of antigen.

Enzyme-linked immunosorbant assay for detection of antibodies against HBsAg

Each well of an Immulon-4 96 well plate (Fisher Scientific) was coated with 500 ng/well of HBsAg 15 (provided by Catherine Wu, University of Connecticut). For binding, HBsAg was suspended in 0.01 M sodium bicarbonate buffer, pH 9.6 and incubated for 60 minutes at 37°C. The wells were washed 5X with phosphate buffered saline containing 20 0.025% Tween 20 (PBS-Tween). Blocking of unreacted sites was accomplished by adding to each well 200 µl of 2.5% bovine serum albumin w/v (Sigma) suspended in PBS; incubation was carried out at 37°C for 1-2 hours. The wells were again washed 5X with 25 PBS-Tween. Sera from each of the animals was initially diluted 1:3 in PBS and 100 µl added to each well. To each of the wells in the vertical row the serum was diluted 3 fold in successive wells. Serum samples and dilutions were incubated for 1 hour at 30 37°C. Each well was again washed 5X with PBS-Tween. To each well 100 µl of a 1:1000 dilution of goat antibody against mouse IgG heavy and light chain,

labeled with horse radish peroxidase (HRP), was added and incubated for 1 hour at 37°C. The wells were again washed 5X with PBS-Tween. Finally, to each well 100 µl of tetramethylbenzidine (TMB) peroxidase 05 substrate (Kirkegard and Perry, Gaithersburg, MD) was added and blue color was allowed to develop for 15-30 minutes. The development of the catalyzed color reaction was evaluated spectrophotometrically with a plate reader.

10 Results

Pre-injection serum samples tested negative for HBsAg. Following intravenous injection of the DNA/ASOR-PL complex, sera from animals were tested for the presence of HBsAg. In 8 out of 10 animals 15 antigen was detected in the sera of animals as early as 3 days or as late as 7 days after injection. All animals which tested positive for the presence of HBsAg became negative for HBsAg 7-10 days after first detection antigen. Sera from these animals were then 20 tested for the presence of antibodies against HBsAg. The antibody titers are presented in Figure 1 as the reciprocal dilution of the sera which was above background levels detected by the plate reader. The results in Figure 1 show that 8 out of 10 animals 25 developed antibodies against HBsAg ranging in relative antibody titer from about 250 to 700. Animal #3 showed an antibody titer of about 50 and in animal #1 no HBsAg or antibody was detected. This data shows that an immune response can be evoked by 30 method of this invention.

-18-

It should be noted that animal #3 tested on two separate occasions (days 4 and 10 after injection of DNA/ASOR-PL) remained negative for HBsAg, but developed antibodies against HBsAg. The ELISA test  
05 for HBsAg has a sensitivity of about 5 pg. Typically, larger amounts of exogenously administered immunogens are required to evoke an immune response in an organism. This suggests that the introduction of a foreign protein via endogenous production in  
10 host cells may be a more efficient process for evoking an immune response than by injecting foreign protein directly into animals.

Equivalents

Those skilled in the art will recognize, or be  
15 able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

Claims

1. A soluble molecular complex for targeting a gene encoding an immunogenic protein or polypeptide to a specific cell, the complex comprising  
05 nucleic acid containing an expressible gene encoding the immunogenic protein or polypeptide complexed with a carrier of a cell-specific binding agent and a gene-binding agent.
2. A soluble molecular complex of claim 1, wherein  
10 the expressible gene is DNA.
3. A soluble molecular complex of claim 1, wherein the immunogenic protein or polypeptide is a component of a pathogen.
4. A soluble molecular complex of claim 1, wherein  
15 the gene-binding agent is a polycation.
5. A soluble molecular complex of claim 4, wherein the polycation is polylysine.
6. A soluble molecular complex of claim 1, wherein  
20 the cell-specific binding agent binds a surface receptor of the cell which mediates endocytosis.
7. A soluble molecular complex of claim 6, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.

-20-

8. A soluble molecular complex of claim 7, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.
9. A soluble molecular complex of claim 1, wherein 05 the expressible gene is complexed with the gene-binding agent by a noncovalent bond.
10. A soluble molecular complex of claim 1, wherein the cell-specific binding agent is linked to the gene-binding agent by a covalent bond.
- 10 11. A soluble molecular complex of claim 1, wherein the expressible gene is complexed with the gene-binding agent so that the gene is released in functional form under intracellular conditions.
- 15 12. A therapeutic composition comprising a solution of the molecular complex of claim 1 and a physiologically acceptable vehicle.
13. A soluble molecular complex for targeting a gene 20 encoding an immunogenic protein or polypeptide to a hepatocyte, the complex comprising an expressible gene encoding the immunogenic protein or polypeptide complexed with a carrier of a ligand for the asialoglycoprotein receptor and a polycation.

-21-

14. A soluble molecular complex of claim 13, wherein the immunogenic protein or polypeptide is a component of a pathogen.
15. A soluble molecular complex of claim 14, wherein the immunogenic protein or polypeptide is hepatitis B surface antigen.  
05
16. A soluble molecular complex of claim 13, wherein the polycation is polylysine.
17. A soluble molecular complex of claim 13, wherein the gene is contained in an expression vector along with genetic regulatory elements necessary for expression of the gene by the hepatocyte.  
10
18. A soluble molecular complex of claim 17, wherein the expression vector is a plasmid or viral DNA.
- 15 19. A method of immunization comprising administering to an organism a soluble molecular complex comprising an expressible gene encoding an immunogenic protein or polypeptide complexed with a carrier of a cell-specific binding agent  
20 and a gene-binding agent, in an amount sufficient to elicit an immune response against the immunogenic protein or polypeptide in the organism.
20. A method of claim 19, wherein the expressible gene is DNA.  
25

-22-

21. A method of claim 19, wherein the immunogenic protein or polypeptide is a component of a pathogen.
22. A method of claim 19, wherein the gene-binding 05 agent is a polycation.
23. A method of claim 21, wherein the polycation is polylysine.
24. A method of claim 19, wherein the cell-specific binding agent binds a surface receptor of the 10 cell which mediates endocytosis.
25. A method of claim 24, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.
26. A method of claim 25, wherein the ligand is an 15 asialoglycoprotein and the targeted cell is a hepatocyte.
27. A method of claim 19, wherein the molecular complex is administered intravenously.

28. A method of producing polyclonal antibodies, comprising:

05       a) administering to an organism a soluble molecular complex comprising an expressible gene encoding an immunogenic protein or polypeptide complexed with a carrier of a ligand for the asialoglycoprotein receptor and a polycation, in an amount sufficient to elicit an immune response against the 10 immunogenic protein or polypeptide in the organism; and

15       b) obtaining antisera from the organism.

29. A method of claim 28, wherein the immunogenic protein or polypeptide is a component of a 15 pathogen.

30. A method of claim 28, wherein the polycation is polylysine.

31. A method of producing monoclonal antibodies, comprising:

20       a) administering to an organism a soluble molecular complex comprising an expressible gene encoding an immunogenic protein or polypeptide complexed with a carrier of a ligand for the asialoglycoprotein receptor 25 and a polycation, in an amount sufficient to elicit an immune response against the immunogenic protein or polypeptide in the organism;

- 05                   b) obtaining antibody-producing cells from the organism;
- c) fusing the antibody-producing cells with immortalizing cells to produce hybridoma cells; and
- d) selecting hybridomas which produce antibodies specific for the immunogenic protein or polypeptide.

10                   32. A method of claim 31, wherein the immunogenic protein or polypeptide is a component of a pathogen.

15                   33. A method of claim 31, wherein the polycation is polylysine.

                  34. Use of a soluble molecular complex according to claim 1 for the manufacture of a medicament for use in immunization against the immunogenic protein or polypeptide.

20                   35. Use according to claim 34, wherein the expressible gene is DNA.

                  20 36. Use according to claim 34, wherein the immunogenic protein or polypeptide is a component of a pathogen.

25                   37. Use according to claim 36, wherein the immunogenic protein or polypeptide is hepatitis B surface antigen.

                  38. Use according to claim 34, wherein the gene-binding agent is a polycation.

-25-

39. Use according to claim 37, wherein the polycation is polylysine.
40. Use according to claim 34, wherein the cell-specific binding agent binds a surface receptor of the cell which mediates endocytosis.  
05
41. Use according to claim 40, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.
42. Use according to claim 41, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.  
10
43. Use according to claim 34, wherein the molecular complex is administered intravenously.

1/1

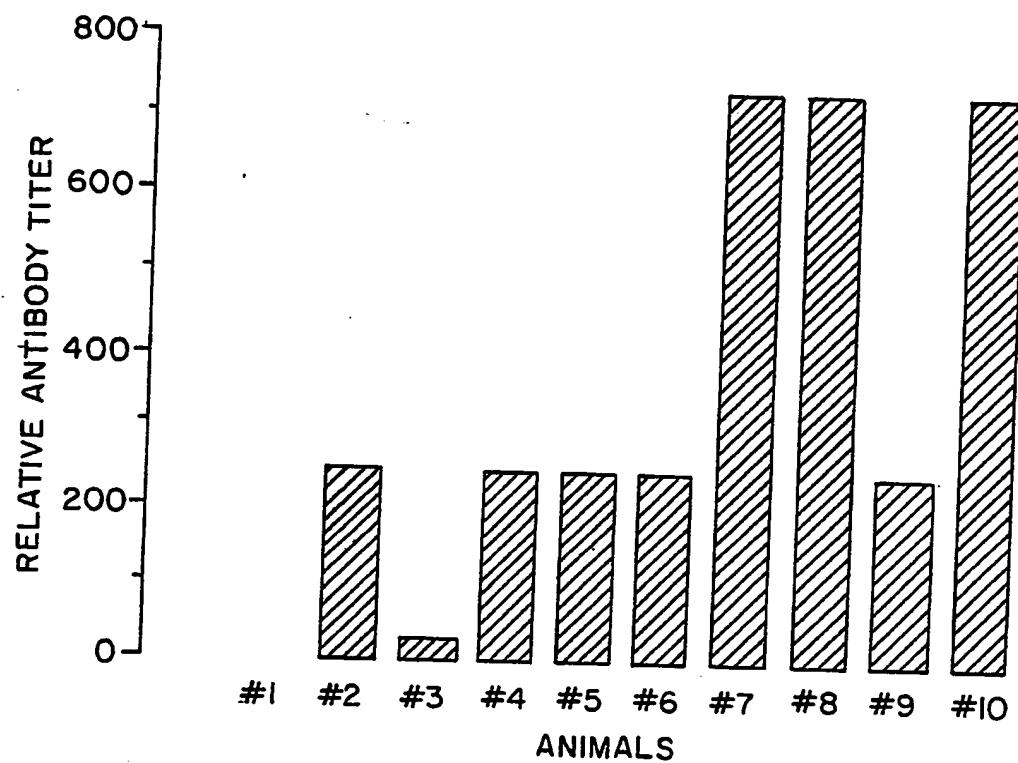


FIG. I

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**